Repression of *sigK* Intervening (*skin*) Element Gene Expression by the CI-Like Protein SknR and Effect of SknR Depletion on Growth of *Bacillus subtilis* Cells[∇]

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Received 1 June 2010/Accepted 23 September 2010

The *Bacillus subtilis* phage DNA-like *sigK* intervening (*skin*) element (48 kb) is excised from the chromosome by DNA rearrangement, and a composite gene, *sigK* (*spoIIIC* and *spoIVCB*), is created on the chromosome during sporulation. In this study, we first focused on the role of *sknR* (*skin* repressor), which has homology with the gene encoding the Xre repressor of defective phage PBSX. The depletion of SknR caused overexpression of the region between *yqaF* and *yqaN* (the *yqaF-yqaN* operon) and a growth defect in *B. subtilis*. Point mutation analysis and an electrophoretic mobility shift assay (EMSA) suggested that SknR functions as a negative regulator of gene expression in the *yqaF-yqaN* operon of the *skin* element through direct interaction with operators of 2-fold symmetry located in the intergenic region between *sknR* and *yqaF*. Deletion analysis revealed that the lethal effect of depletion of SknR was related to overexpression of *yqaH* and *yqaM*, whose products were previously reported to associate with DnaA and DnaC, respectively. Furthermore, overexpression of either *yqaH* or *yqaM* caused cell filamentation and abnormal chromosome segregation, which suggested that overproduction of these proteins inhibits DNA replication. Moreover, overexpression of *yqaM* inhibited the initiation of replication. Taken together, these data demonstrate that the *B. subtilis* *skin* element carries lethal genes, which are induced by the depletion of *sknR*.

The genome of *Bacillus subtilis* strain 168 contains two prophages, SP β and PBSX (a defective prophage), and eight prophage-like regions (pro Φ -1 to pro Φ -7 and the *skin* element) (14, 39, 42, 43). These resident genomes comprise 185 (SP β), 42 (PBSX), 61 (*skin*), 24 (pro Φ 2), 20 (pro Φ 1), and 10 (pro Φ 3) genes (41). Strain 168 is lysogenic for phage SP β , which is induced by treatment with mitomycin C (MMC) (20). In contrast, PBSX is a defective phage (34) that directs the formation of phage particles containing randomly selected host chromosomal DNA in the heads (28). pro Φ 2 was recently characterized as a mobile genetic element, ICEBs1, that can excise from the chromosome and transfer to recipient cells by the SOS response and intercellular signaling (2). In addition, ϕ 105 is a well-characterized temperate bacteriophage that infects *B. subtilis* with a unique chromosomal attachment site in the *B. subtilis* genome, and the ϕ 105 prophage, like SP β , is strongly induced by treatment with MMC (29).

Excision of the *skin* (sigma $\mathbf{K}$ intervening) element from the chromosome leads to reconstitution of the *sigK* (*spoIIIC* and *spoIVCB*) gene, which encodes the mother-cell-specific $\sigma^{\mathbf{K}}$ factor during sporulation (13, 32, 38). This excision event requires a site-specific DNA recombinase, which is encoded by *spoIVCA* located in the 48-kb *skin* element (13, 32). The *spoIVCA* gene is controlled specifically in the mother cell by

the mother-cell-specific sigma factor $\sigma^{\mathbf{E}}$, indicating that the effects of the DNA rearrangement should not be inherited by the next generation, since the mother cell is discarded after maturation of the forespore (30, 32). Interestingly, the presence of an intact rearranged *sigK* gene in the mother cell has no effect on sporulation (13, 31).

The length of the *skin* element is 48 kb, which corresponds to 1% of the entire *B. subtilis* genome (39). The *skin* element includes genes known to function in arsenite and arsenate resistance (*ars* operon [33]), a quorum-sensing system (*rapE-phrE* [10]), a peptidoglycan hydrolase (*cwlA* [7, 15]), and a toxin-antitoxin module (*txpA-ratA* [37]). However, the *skin* element is assumed to be a cryptic remnant of an ancestral prophage or phage-like element, since the majority of genes in the *skin* element are thought to encode proteins involved in phage formation (39). Possible functions of these phage-related open reading frames (ORFs) in the *skin* element are the phage endonuclease, terminase, capsid protein, cell wall hydrolase (7, 15), and holin (39).

We first focused on the role of *sknR* (*skin* repressor), which has homology with the gene encoding the Xre repressor of defective phage PBSX. The *yqaF* gene is located 176 bp upstream of the *sknR* gene, and there is no ambiguity about the direction of transcription, though it might not have been known prior to this study whether or not *yqaF* is transcribed. SknR and YqaF also have homology with ϕ 105 proteins: the immunity repressor c ϕ 105 (ORF1) and the ORF4 product (which encodes the Cro-like protein), respectively. The gene organization of the *skin* element also is relatively similar to that of ϕ 105 (39). The most well-defined model system for the induction of temperate phage is the *Escherichia coli* λ phage.

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[§] Supplemental material for this article may be found at <http://jb.asm.org/>.

[∇] Published ahead of print on 1 October 2010.

Lysogeny is maintained by CI, which represses the signals for the onset of the lytic pathway (6). Similarly, in $\phi 105$, the $c\phi 105$ protein represses the promoter for the lytic cycle (40). It has been shown that SknR is a probable transcriptional regulator (phage related) (BSORF web site, <http://bacillus.genome.ad.jp/>), but there has been no investigation of the regulation by SknR and the effects of its deregulation.

In this report, we present evidence that SknR has a CI-like function in *B. subtilis* and that depletion of SknR causes growth inhibition through overexpression of the region between *yqaF* and *yqaN* (the *yqaF-yqaN* operon), which carries the genes *yqaH* and *yqaM*, whose products affect DNA replication.

MATERIALS AND METHODS

Bacterial strains, plasmids, and general methods. The oligonucleotide primers used for PCR amplification are listed in Table S1 in the supplemental material. Table S2 in the supplemental material lists the strains and plasmids used in this study. *B. subtilis* transformation was performed according to the standard method described by Davidoff-Abelson and Dubnau (5). Plasmids were constructed in *E. coli* strain JM105 or MC1061, unless otherwise indicated.

Plasmid and strain construction. To construct pISyqaf-lacZ(-184) and pISyqaf-lacZ(-176), DNA fragments that corresponded to nucleotides (nt) -184 to +10 of *yqaF* and -184 to -37 of *yqaF* were amplified with primer pairs yqAFFE-yqaFRH and yqAFFE-yqaFDRH8. The PCR product and pIS284, which delivers promoter-*E. coli lacZ* fusion to the *B. subtilis amyE* locus and carries EcoRI and HindIII sites in that order upstream of *lacZ*, were completely digested with EcoRI and HindIII and ligated.

To construct pISyqaf-lacZ(-98), pISyqaf1A-lacZ(-98), pISyqaf2C-lacZ(-98), pISyqaf3C-lacZ(-98), pISyqaf4C-lacZ(-98), pISyqaf5A-lacZ(-98), pISyqaf6A-lacZ(-98), pISyqaf7C-lacZ(-98), pISyqaf8C-lacZ(-98), pISyqaf10A-lacZ(-98), pISyqaf12C-lacZ(-98), pISyqaf14C-lacZ(-98), and pISyqaf10A-lacZ(-98), DNA fragments of *yqaF* (nt -98 to -1) were amplified with forward primer FPMFE or the mutated forward primer (mutated forward primers were designated FPMFE1A to FPMFE1Z; see Table S1 in the supplemental material) and reverse primer FPMRH. The PCR product and pIS284 were completely digested with EcoRI and HindIII and then ligated. Before transformation into *B. subtilis*, all pIS plasmids were linearized by digesting with SacI.

Integrational plasmids pMUTin(*Pspac-sknR*), pMUTin(*Pspac-yqaH-N*), pMUTin(*Pspac-yqaM*), and pMUTin(*Pspac-yqaN*) carrying a DNA fragment of *sknR* (nt -16 to +163), *yqaH* (nt +1 to +90), *yqaM* (nt +1 to +456), or *yqaN* (nt -23 to +260) were amplified with primer pairs sknRpF-sknRpR, yqHFHSD-yqaHRB, yqMFHSD-yqMRB, and yqNFH-yqNRB2, respectively. The primer yqMFHSD contains the SD sequence of *yqaL*. The amplified PCR product was completely digested with HindIII and BamHI and ligated into the corresponding sites of pMUTinT3 (27).

To construct the deletion strains (see Fig. 4), 5' flanking regions of each deleted part were amplified with primer pairs arsCFN-arsCR for YA11, yqbMFN-yqbMR for YA12 and YA15, yqbCFN-yqbCR for YA14 and YA16, and yqxJFN-yqxJR for YA13, YA17, YA18, YA19, TK01, TK02, and TK03, and then the PCR products were completely digested with NheI. Then 3' flanking regions of each deleted part were amplified with primer pairs yqbNF-yqbNRB for YA13, yqAFF-yqaFRB for YA11, YA12, and YA14, yqaPF-yqaPRB for YA15 and YA16, yqbIF-yqbIRB for YA17, yqbDF-yqbDRB for YA18, yqaSF-yqaSRB for YA19, yqNF-yqNRB for TK01, yqaIF-yqaIRB for TK02, and yqHF-yqHRB for TK03, and the PCR products were completely digested with BamHI. The coding region of the *spc* gene from pKL147 (17) was amplified with the primer pair spcFB-spcRN, and then the products were completely digested with BamHI and NheI. The three digested DNA fragments were ligated, the ligation product was used as a template for PCR amplification using primers arsCFN-yqaFRB for YA11, yqbMFN-yqaFRB for YA12, yqxJFN-yqbNRB for YA13, yqbCFN-yqaFRB for YA14, yqbMFN-yqaPRB for YA15, yqbCFN-yqaPRB for YA16, yqxJFN-yqbIRB for YA17, yqxJFN-yqbDRB for YA18, yqxJFN-yqaSRB for YA19, yqxJFN-yqNRB for TK01, yqxJFN-yqaIRB for TK02, and yqxJFN-yqHRB for TK03, and then the PCR products were used for *B. subtilis* transformation to construct the respective deletion mutants.

To construct pET32asknR, a *sknR* fragment was amplified by PCR with oligonucleotide primers sknRPF and sknRPRH. The PCR product was digested with BamHI and HindIII and ligated with pET-32a digested with the same enzymes. The resulting plasmid, pET32asknR, was cloned into *E. coli*

BL21(DE3)/pLysS. All plasmid constructions were sequenced to confirm the absence of mutations in the cloned fragment.

Growth conditions and β -galactosidase assay. Cells were grown in Difco sporulation medium at 37°C. The β -galactosidase activity was determined as previously described by Miller (22) using *o*-nitrophenyl- β -D-galactopyranoside as the substrate. Specific activity was calculated relative to the optical density at 600 nm (OD₆₀₀) of each sample and was expressed in nanomoles of substrate hydrolyzed per milligram per minute.

Isolation of the overexpressed His-SknR (SknR) protein. *E. coli* BL21(DE3)/pLysS harboring pET32asknR (strain EPI) was incubated overnight at 30°C in LB medium supplemented with ampicillin (50 μ g/ml), and 1 ml of the culture was used to inoculate 100 ml of the same medium. Cells were grown at 37°C until the optical density at 600 nm of the culture reached ~0.5. IPTG (isopropyl- β -D-thiogalactopyranoside) was then added to the culture at a final concentration of 1 mM. After incubation for 4 h, cells were harvested by centrifugation (5,000 $\times$ g) at 4°C and washed with lysis buffer (50 mM NaH₂PO₄ $\cdot$ 2H₂O, 300 mM NaCl, 10 mM imidazole) and 100 μ M phenylmethylsulfonyl fluoride. Cells were treated with 1.5 ml of lysozyme (10 mg/ml) on ice, frozen in liquid nitrogen, thawed on ice, and immediately sonicated. The sonicated cells were treated with 10 μ l of DNase I (70 U/ μ l) and 37.5 μ l of RNase A (2 mg/ml). Cleared lysates were then mixed with 2.5 ml of Ni-nitrilotriacetic acid (Qiagen) affinity resin. The Ni-nitrilotriacetic acid column was washed using 20 mM imidazole, and proteins were eluted with 300 mM imidazole. Purified SknR-His protein possessed a Trx-His 6 leader that was removed by incubation with enterokinase. The eluted protein was then dialyzed against buffer A (1 M NaCl, 5 mM MgCl₂, 50 mM Tris HCl [pH 7.8]) and stored with 10% glycerol at -20°C. The protein concentration was determined by the Bio-Rad protein assay.

EMSA. The DNA fragment for electrophoretic mobility shift assays (EMSA) was labeled with digoxigenin by using the 5' digoxigenin-labeled primer T3UP-DIG (5' end, +93 of *lacZ*). Probe C was prepared using the chromosomal DNA of FZW and oligonucleotide primers yqAFDFE6 and yqAFDFE9 as forward primers and T3UP-DIG as a reverse primer. Probe 1A was prepared using the chromosomal DNA from strain 1A and oligonucleotide primers yqAFDFE6 and T3UP-DIG. Probes 2 and 7 were prepared using the chromosomal DNA of FD8 and primers yqAFDFE5 and yqAFDFE7 as forward primers and T3UP-DIG as a reverse primer. In each reaction, the described concentrations of protein and DNA were incubated in buffer [25 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (pH 6.1), 25 mM NaCl, 0.5 mM dithiothreitol, 2 mM EDTA, 2 mM MgCl₂, 5% glycerol] for 15 min at room temperature. The samples were loaded onto a 6% polyacrylamide gel in Tris-borate-EDTA. After a prerun of ~1 h, the gel was electrophoresed at 4°C for 150 min and was blotted on an N⁺ Hybond nylon membrane overnight. The digoxigenin-labeled DNA fragment bound to antidigoxigenin-alkaline phosphatase conjugate (1:5,000 dilution) was detected with nitroblue tetrazolium/BCIP (5-bromo-4-chloro-3-indolylphosphate) (according to the protocol described by Roche Applied Chemicals).

Fluorescence microscopy. Cells were grown at 37°C in LB medium. DNA was stained by DAPI (4',6-diamidino-2-phenylindole) before the observation. The cells were viewed by microscopy using an Olympus BX50 microscope with a 100 $\times$ UplanApo objective lens. Photos were captured by Metamorph, version 6.1, software (Universal Image).

RT-PCR. Strain SKNRp was grown in LB medium in the presence or absence of 1 mM IPTG at 37°C, and samples were removed for analysis at the end of the exponential phase of growth. Total RNA was extracted from the cells and treated with DNase I (TaKaRa) to remove chromosomal DNA. Reverse transcriptase PCR (RT-PCR) was performed using 1 μ g of total RNA and a PrimeScript RT-PCR Kit (TaKaRa) according to the manufacturer's instructions. cDNA was amplified using *Ex taq* HS from the kit. mRNA encoding ribosomal protein S18 (*rpsR*) was used as a control of total RNA. Primer pairs rpsRF-rpsRR, yqAFF2-yqAFAR, yqAFF2-yqMRB3, and yqMFH-yqNRB3 were used to amplify *rpsR*, *yqaF-yqaA*, *yqaF-yqaM*, and *yqaM-yqaN*, respectively (see Table S1 in the supplemental material).

Southern blot analysis. Total cellular DNA was digested with HindIII, and fragments were separated on an 0.8% agarose gel, transferred by capillary transfer to a Hybond-N⁺ membrane (GE Healthcare), and probed with *oriC* (amplified with dnaA-F and dnaA-F primers) and *terC* (amplified with desR-F and desR-R primers) PCR products, which hybridize to 2,151-bp and 629-bp chromosomal HindIII fragments, respectively. Probes were labeled with DIG-11-dUTP dATP using the DIG DNA labeling kit (Roche). The *oriC/terC* ratio was determined by measuring the intensities of hybridization to the 2,151-bp and the 629-bp fragments using ImageJ software (<http://rsb.info.nih.gov/ij/index.html>). Hybridization signals were normalized to the signals of control spore DNA included on the gel, where *oriC* and *terC* bands are present in a 1:1 ratio.

FIG. 1. Severe growth inhibition of the *Pspac*-controlled *sknR* strain without IPTG. (A) Genetic construction of the IPTG-inducible *sknR* strain (designated SKNRp) and *skin*-less strain SL. *Pspac*, *spac* promoter. *PsigK*, *sigK* promoter. (B) Growth properties of strain SKNRp without and with IPTG (1 mM) and *skin*-less strain SL. Strains were grown on LB solid medium at 37°C.

RESULTS

***sknR* is an essential gene in the *skin* element.** Within the framework of the *B. subtilis* functional genomics project conducted by a Japanese and European consortium (11, 27), we identified a growth-deficient *yqaE* (renamed *sknR*) mutant, since no transformants were detected by simple insertional mutagenesis to obtain a *sknR* mutant. We therefore constructed a conditional SKNRp (*Pspac-sk nR*) strain in which the *sknR* gene was under the control of the LacI-repressible, IPTG-inducible *Pspac* promoter. Growth of SKNRp in the absence or presence of IPTG was determined on LB solid medium. As shown in Fig. 1, a severe growth defect was observed in *Pspac-sk nR* cells in the absence of IPTG, whereas normal growth was observed in the presence of IPTG, suggesting that *sknR* is essential for cell growth. It has been shown previously that a *skin*-less strain (with no *skin* element but with an intact *sigK* gene) exhibits normal growth and sporulation phenotypes (13, 31). We too observed a normal growth phenotype of a *skin*-less strain (strain SL, no *sknR*, Fig. 1). These results suggested that *sknR* is not actually an essential gene in *B. subtilis* but rather that some genes regulated by SknR in the *skin* element are involved in the inhibition of cell growth.

SknR negatively influences transcription from *yqaF-yqaN* operon in the *skin* element. The *sknR* gene is located in the *B. subtilis* *skin* element, which is positioned between *spoIVCB* and *spoIIIC* and is excised during sporulation by DNA rearrangement to create an intact *sigK* (*spoIVCB-spoIIIC* fused) gene. SknR shares 30% homology with the Xre repressor, which maintains the lysogenization of the *B. subtilis* defective prophage PBSX (19) and is functionally equivalent to the *E. coli* λ CI repressor.

To determine the regulatory effects of *sknR* in the *skin* element, transcriptional analysis was performed of four possible promoters in a candidate target region near *sknR*, located

FIG. 2. Significant expression of *yqaF-lacZ* in the *sknR*-depleted strain. (A) Organization of open reading frames (between *spoIIIC* and *yqaR*) in the *skin* element. The 176-bp and 197-bp intergenic regions between the head-to-head genes *sknR* and *yqaF* and *yqaP* and *yqaQ*, respectively, are shown. The regions shown by thick bars were analyzed by RT-PCR. The circle indicates the predicted terminator sites. (B) β -Galactosidase activities of the *PyqaF-lacZ* strains with or without the *skin* element. Closed circles, FZW (*PyqaF-lacZ*); open circles, FZS (*PyqaF-lacZ* Δ skin). (C) RT-PCR analysis of expression of *yqaF* through *yqaN*. RNA from *Pspac-sk nR* cells was isolated in the presence of 1 mM IPTG (+) or in the absence of IPTG (-). *rpsR* (control), *yqaF-yqaA*, *yqaF-yqaM*, and *yqaM-N* expression were examined by RT-PCR. All samples were analyzed on 1% agarose gels stained with ethidium bromide.

between head-to-head genes (a 176-bp region between *sknR* and *yqaF* and a 197-bp region between *yqaP* and *yqaQ*) (Fig. 2A). The intergenic regions were amplified, fused in each orientation to the *lacZ* reporter gene, and introduced into the *amyE* locus in strain 168 (strain FZW, SknR⁺) and a *skin*-less strain (strain FZS, SknR⁻). Differences in expression patterns between the 168 and *skin*-less strains were examined. As shown in Fig. 2B, expression of *PyqaF-lacZ* was very low in strain 168; however, it increased approximately 1,000-fold in the *skin*-less strain. No or very low expression was observed in *PsknR-lacZ*, *PyqaP-lacZ*, and *PyqaQ-lacZ* from both the wild-type and *skin*-less backgrounds (data not shown). These results indicated that the depletion of SknR leads to significant expression from *PyqaF*. In addition, expression of *sknR* appears to be maintained at a low level, indicating that a small amount of SknR is sufficient to repress transcription from *PyqaF*.

To evaluate the presence of transcripts in the *Pspac-sk nR* strain, we used RT-PCR to analyze the region between *yqaF* and *yqaN*, which is followed by a ρ -independent terminator-like structure (Fig. 2A). RNAs were purified from both *Pspac-sk nR* cells in the presence (SknR⁺) and absence (SknR⁻) of

IPTG at the end of the exponential growth phase, and the presence of transcripts corresponding to genes in the *skin* element was tested by RT-PCR. The primers that were used are listed in Table S1 in the supplemental material, and the results are given in Fig. 2C. A single thick band of the expected size was obtained when the *yqaF-yqaD* region was amplified from RNA purified from *SknR*[−] cells, but no band for this region was obtained from *SknR*⁺ cells (Fig. 2C). Single bands of the expected sizes were found when using primer pairs for amplification of *yqaF-yqaD*, *yqaF-yqaM*, and *yqaM-yqaN*, indicating that *yqaF* through *yqaN* (named the *yqaF-yqaN* operon) are cotranscribed *in vivo*.

SknR binds to the operators, which have 2-fold symmetry. The nucleotide sequence of the intergenic region between *sknR* and *yqaF* indicated that a candidate promoter sequence for *yqaF* (5'-TTGACA-17 bp-TATTAT-3' at −72 to −44, relative to the initiation codon of *yqaF*) is very similar to the consensus sequence (5'-TTGACA-17 bp-TATAAT-3') recognized by σ^A (Fig. 3A). In addition, three possible regulator-binding sites (OR1, OR2, and OR3) are found in the same region; they contain 2-fold symmetry (5'-GTAACGAATCGA TAC-3', in the case of OR2) (Fig. 3A). The OR2 site is located from −64 to −50 (relative to the initiation codon of *yqaF*) between the −35 box and the −10 box. To examine whether the operators (ORs) are direct targets for SknR-mediated repression, we performed electrophoretic mobility shift assays (EMSA) using the SknR protein, which was purified following overexpression in *E. coli*, and probe 7 (containing OR2), probe C (OR2 and OR3), and probe 2 (OR1 and OR2) to the region between *sknR* and *yqaF* (Fig. 3B). A DNA fragment carrying the pIS284 vector region, including no ORs, was used as a negative control, which resulted in no shifted bands (data not shown). The EMSA result using probe 7 showed one shifted band with SknR, suggesting that this fragment has a single SknR-binding site (OR2) (Fig. 3C). The results using probe C showed a two-step binding process: a large first shift at 0.74 μ M SknR and a second slight shift at 3.7 μ M (Fig. 3C), suggesting that two binding sites (OR2 and OR3) are contained in this fragment. We also used probe 1A, which contains a single-base substitution in OR2 (G→A; the first nucleotide of the 2-fold symmetry in OR2). The EMSA results with probe 1A found only one shifted band, whose position was identical with that of the shifted band using probe C at 0.74 μ M SknR (Fig. 3C). Therefore, the SknR-OR3 complex forms at 0.74 μ M SknR and SknR-OR2 complex does not. We further examined the SknR-binding activity using probe 2 (OR1 and OR2) and observed a broad band and several higher shifted bands, which probably implies that SknR binds to OR1 or OR2 at 0.74 μ M concentration, and it binds to both OR1 and OR2 at 3.7 μ M concentration. Interestingly, the higher shifted bands seemed to converge to a band at a position between the broad and the higher bands at 3.7 μ M, suggesting that SknR binds to more than one site and forms a distinct higher-order structure. These results suggested that OR1- and OR2-bound SknR interact with one another. Overall, these results demonstrated that SknR binds directly to the three ORs (OR1, OR2, and OR3) located in the intergenic region between *sknR* and *yqaF*.

To further analyze which positions of the 2-fold symmetry of the operator are important for control of expression by SknR, we substituted each position of the symmetrical region in OR2.

FIG. 3. Three operator sequences (OR1, OR2, and OR3) in the regulatory region of *yqaF* serve as a specific recognition site for SknR binding, and increased expression is observed in strains harboring OR2 point mutations. (A) Nucleotide sequence of the intergenic region between the head-to-head genes *sknR* and *yqaF*. The initiation codon and predicted −10 and −35 regions are shown in boldface uppercase. The lines show the imperfect palindrome sequences (ORs). (B) Structural representation of the upstream region of the intergenic region between *sknR* and *yqaF* and the probe DNA fragments used in the assay. The nucleotide positions are indicated relative to the initiation codon of *yqaF*. (C) EMSA with probes 7, C, 1A, and 2. The DNA probes (5 ng/ μ l) were incubated with increasing amounts of SknR protein (0, 0.15, 0.74, and 3.7 μ M) and analyzed by EMSA. The arrows show the positions of the protein-DNA complex, and expected SknR-bound ORs are indicated. (D) Mutagenesis analysis of the OR2 sequence. Mutated nucleotides harbored in each strain are shown in boldface uppercase. Culture samples were assayed for β -galactosidase activity. The arrow shows the imperfect palindrome sequences.

FIG. 4. Identification of genes that cause growth inhibition in *sknR*-depleted cells by long-distance deletion of the *skin* element. The deleted region of each strain is shown by a boxed bar. The relevant genes that are represented in the *skin* element between *spoIVCB* and *spoIIC* are shown. All deletion strains were constructed in the SKNRp (*Pspac-sknR*) strain background, and growth was observed in the *sknR*-depleted condition (no IPTG) on LB solid medium at 37°C. (–), growth inhibition was observed; (+), normal growth was observed. Asterisks indicate positions of terminators.

A point mutation was introduced into the sequence, and promoter activity of the region –98 to –1 with a mutated OR2 was analyzed using a *lacZ* fusion construct. Activity of the mutated promoter was greatly increased over that of the non-mutated promoter (CW) (Fig. 3D). The ratio of the increase was calculated by dividing the β -galactosidase activity of strains harboring a mutated promoter by that of strains harboring a native promoter, as follows: 1A, 1,110%; 2C, 986%; 3C, 490%; 4C, 864%; 5A, 690%; 6A, 147%; 7C, 133%; 8C, 151%; 10A, 879%; 12C, 28%; and 14C, 155%. These results suggested that at least positions 1, 2, 3, 4, 5, and 10 are important for repression, whereas positions 6, 7, 8, 12, and 14 are less important for repression. Moreover, strain DZ harboring the *lacZ* fusion with mutations in 1A, 2C, 3C, 4C, 5A, and 10A was also investigated, resulting in the highest activity (1,200%) that was measured in the *skin*⁺ background. Thus, mutations in the left half of the inverted repeat seemed to be more important, but position 10 in the right half was an exception. These results indicate that the SknR may not simply bind symmetrically as a homodimer or tetramer in the inverted repeat sequence, or the binding of the SknR to each half site may be asymmetrical.

Taken together, our data indicate that SknR functions as a negative regulator for gene expression of genes in the *skin* element, through direct interaction with 2-fold symmetry sequences OR2 and likely the other ORs (OR1 and OR3) located in the intergenic region between *sknR* and *yqaF*, and that derepression of SknR causes significant expression of the *yqaF-yqaN* operon.

Genes located in the *skin* element can affect cell growth. We have shown a severe growth defect phenotype of *Pspac-sknR* cells in the absence of IPTG, suggesting that the lethal gene controlled by SknR is localized to the *skin* element. To screen for such a gene, we next performed long deletion analysis, since the (potent) toxic genes (e.g., *yqxH* [encoding the potent holin] and *txpA* [encoding the peptide toxin] [37]) are dispersed in the *skin* element (Fig. 4). If the region including the lethal gene is deleted, lethality due to the repression of *sknR* in

FIG. 5. Growth inhibition caused by overexpression of genes in the *skin* element. Wild-type strain 168, YQAHNp (HN, *Pspac-yqaH*), YQAHp (H, *Pspac-yqaH* $\Delta yqaI-yqxJ$), YQAMNp (MN, *Pspac-yqaMN*), YQAMp (M, *Pspac-yqaM* $\Delta yqaN-yqxJ$), and YQANp (N, *Pspac-yqaN*) were incubated on LB solid medium at 37°C for 7 h with or without 1 mM IPTG.

the absence of IPTG will be suppressed. We constructed a deletion series from the *spoIVCB* side and from the *spoIIC* (*yqaF*) side of the *skin* element in the SKNRp (*Pspac-sknR*) strain background and then examined cell growth on LB plates in the *sknR*-depleted condition (without IPTG). The deletion series from the *spoIVCB* side showed that deletions spanning from *arsC*, *yqbM*, or *yqbC* to *yqaF* (YA11, YA12, and YA14) had no impact on cell growth (lethality was suppressed). However, deletions spanning from *yqbM* or *yqbC* to *yqaP* (YA15 and YA16) resulted in severe growth inhibition (lethality was not suppressed). The location of the lethal gene was further delineated by analyzing a deletion series from the *spoIIC* (*yqaF*) side. The deletions from *yqaI*, *yqaN*, *yqaS*, *yqbD*, *yqbI*, and *yqbN* to *yqxJ* (TK02, TK01, YA19, YA18, YA17, and YA13) resulted in severe growth inhibition (lethality was not suppressed), whereas the deletion from *yqaH* to *yqxJ* (TK03) had no effect on cell growth (lethality was suppressed) (Fig. 4). The results of the deletions suggested that *yqaH* alone is sufficient to cause the growth defect, but note that they do not negate the possibility that other genes related to the lethality could be located in the *skin* element. As shown in Fig. 2B, depletion of *sknR* leads to a significant level of expression of the *yqaF-yqaN* operon. Therefore, we anticipated that the growth defect was caused by overexpression of the *yqaF-yqaN* operon.

To examine our hypothesis, we next constructed a *Pspac-yqaH-yqaI-yqaJ-yqaK-yqaL-yqaM-yqaN* strain (YQAHNp) by introducing pMUTin(*Pspac-yqaH*) upstream of *yqaH*. The lethality of YQAHNp was then examined by induction of *yqaH-yqaN* directly from the *Pspac* promoter in the presence of 1 mM IPTG. As expected, lethality was observed in the IPTG-induced strain (Fig. 5, HN, IPTG⁺), whereas normal growth was observed in the IPTG[–] strain (Fig. 5, HN, IPTG[–]). This result supports the evidence presented above that the growth defect of the SknR-depleted strain is caused by overexpression of the *yqaF-yqaN* operon. We further examined the cytological features of the overexpressing strain, YQAHNp. Samples of wild-type (strain 168) and YQAHNp cells were taken from exponential-phase LB liquid cultures and stained with DAPI to visualize cytoplasmic nucleic acids. Phase-contrast and fluorescence microscopy revealed that the cells of 168 and YQAHNp IPTG[–] mutant strains show a long chain of cells (regularly spaced septa in a chain of cells), whereas in 1 mM IPTG, YQAHNp cells appeared to be extremely filamentous (irreg-

FIG. 6. Morphological changes caused by overexpression of genes in the *skin* element. Wild-type strain 168 (WT), YQAHNp (HN, *Pspac-yqaH*), YQAHp (H, *Pspac-yqaH ΔyqaI-yqxJ*), YQAMp (M, *Pspac-yqaM ΔyqaN-yqxJ*), YQAMNp (MN, *Pspac-yqaM*), and YQANp (N, *Pspac-yqaN*) were incubated in LB liquid medium. Cells incubated until 3 h after the addition of 1 mM IPTG to exponentially growing culture were stained with FM4-64 (Membrane) and DAPI (DNA) to visualize cell membranes and DNA, respectively. White bar in WT is 2 μ m.

ular septa in a filament) and the DNA localization was dispersed (Fig. 6, HN, IPTG⁺).

To determine the lethal gene between *yqaH* and *yqaN*, we constructed several strains carrying the *Pspac*-controlled genes in the region, and we found that strain YQAMNp (*Pspac-yqaMN*), carrying the *Pspac*-controlled *yqaM* and *yqaN* genes, also exhibits IPTG-dependent lethality (Fig. 5, MN, IPTG⁺). To further investigate this region, we constructed strains YQAMp (*Pspac-yqaM ΔyqaN-yqxJ*) and YQANp (*Pspac-yqaN*), which express only *yqaM* and *yqaN*, respectively. As shown in Fig. 5 (M and N, IPTG⁺), growth inhibition was observed in YQAMp but not YQANp, indicating that the major factor for lethality is *yqaM*. We further observed the cytological features of the YQAMNp, YQAMp, and YQANp strains. Figure 6 (MN, M, and N, IPTG⁺) reveals filamentous cells and dispersed DNA localization phenotypes in YQAMp and YQAMNp IPTG-induced strains in an LB liquid culture. These results suggested that strong inhibition of nucleoid segregation occurred from the overexpression of *yqaM*.

On the other hand, our long deletion analysis suggested that YqaH also contributes to the lethal phenotype upon SknR depletion (Fig. 4). To examine this possibility, we next constructed strain YQAHp (*Pspac-yqaH ΔyqaI-yqxJ*), which expressed only *yqaH* from the *Pspac* promoter, by introducing *ΔyqaI-yqxJ* from TK02 into YQAHNp (*Pspac-yqaHN*). The cytological properties of YQAHp were to some extent similar to those of YQAHNp: the cells were extremely filamentous and showed a dispersed DNA localization (Fig. 6, H, IPTG⁺), although this strain grew somewhat slowly in the presence of 1

FIG. 7. The *ori/ter* ratio is decreased in the YqaM-overexpressed cells. YQAHp (*Pspac-yqaH ΔyqaI-yqxJ*) and YQAMp (*Pspac-yqaM ΔyqaN-yqxJ*) were grown at 37°C in LB medium. Each culture was diluted in fresh LB medium in either the absence or presence of 1 mM IPTG after reaching an OD₆₀₀ of about 0.5. Cells were collected after 1 h of incubation at 37°C, DNA was extracted and digested with HindIII, and Southern hybridization was performed with an *ori* probe (*dnaA*) and a *ter* probe (*desR*). A sample of spore DNA digested with HindIII was also included on the blot. This sample contains *ori* and *ter* in a 1:1 proportion.

mM IPTG (Fig. 5, H, IPTG⁺). These results revealed that cells carrying the *Pspac*-induced *yqaH* or *yqaM* were frequently filamentous, with irregular positioning of the nucleoid, which suggested that overproduction of these genes inhibits DNA replication.

We next determined the origin-to-terminus ratio for YQAHp (*Pspac-yqaH ΔyqaI-yqxJ*) and YQAMp (*Pspac-yqaM ΔyqaN-yqxJ*) with and without IPTG, respectively. In order to correct for differential labeling efficiencies of the *oriC* and *terC* probes, we included a DNA sample from *B. subtilis* spores in which the *ori/ter* ratio is 1. In the YQAHp and YQAMp cells in the absence of IPTG, the *ori/ter* ratios (2.5 and 2.7, respectively) were significantly higher than the ratio in spores, whereas in YQAMp cells in the presence of IPTG it was found to be 1.0 (Fig. 7). These results suggest that at least overexpression of *yqaM* inhibits initiation of replication but allows ongoing replication to proceed to completion. Overexpressing YqaH cells show filamentous growth and the segregation defects of the nucleoid but had an *ori/ter* ratio that was similar to that of nonoverexpressing cells, suggesting that replication is slowed in this strain.

Taken together, these data indicate that SknR represses transcription of the *yqaF-yqaN* operon. Genes in this operon encode proteins that include homologs of DNA replication proteins and/or proteins that interact with the DNA replication machinery, and therefore depletion of SknR most likely causes inhibition of DNA replication.

DISCUSSION

A number of studies have explored the phage DNA replication system directed by phage-encoded proteins, in which some proteins are homologous to host replication proteins. For example, the host DNA replication proteins DnaC and DnaI have homology with G40P and G39P, respectively, of the SPP1 phage, which is a well-characterized virulent *B. subtilis* double-

stranded DNA (dsDNA) phage. The G40P and G39P proteins have roles in phage DNA replication as a helicase and its loader, respectively (3, 26), while the *skin* proteins YqaH and YqaM are homologous to ORF6 of *B. subtilis* temperate phage ϕ 105 and YkdC of the defective phage PBSX, respectively (23, 39). Meanwhile, it is known that some phage-related genes can affect host DNA replication. The P protein encoded in the *E. coli* λ phage genome associates with the DnaB DNA helicase (the counterpart of the *B. subtilis* DnaC protein) and is lethal to the host because of its interaction with the *E. coli* replication initiation protein DnaA (4). The P protein appears to shut off the host replication system by interacting with DnaB and redirecting DnaB and/or DnaA to perform phage DNA replication. Furthermore, Liu et al. (18) reported that the ORF104 product of *Staphylococcus aureus* phage 77 interacts with DnaI (the host helicase loader for host replication), and expression of ORF104 inhibits cell growth, followed by the inhibition of DNA replication. Of interest, in a previous report, yeast two-hybrid experiments revealed that YqaH and YqaM interact with the replication initiation protein DnaA and with the DNA helicase DnaC, respectively (25). YqaM and YqaL have homology with the helicase loader DnaI and with DnaC, respectively, in the *B. subtilis* chromosome (21). DnaI is a component of the DnaA-dependent primosome assembled at *oriC* and loads the replicative ring helicase DnaC onto the DNA during the priming of DNA replication in *B. subtilis* (9). DnaA binds to the DnaA box near the DNA replication initiation site and unwinds the AT-rich sequence (24). YqaN has homology with a Holliday junction resolvase in *E. coli* encoded by RusA (36). We showed here that overexpression of either *yqaH* or *yqaM* caused cell filamentation and abnormal chromosome segregation, resulting in a marked reduction in viability (Fig. 5 and 6). We further indicated that *yqaM* is involved in the initiation of replication (Fig. 7). Therefore, we propose that overproduction of YqaH and YqaM leads to a perturbation of cellular DNA replication through interactions with and inhibition of DnaA and DnaC, which subsequently causes growth inhibition. However, it remains to be determined how the initiation of replication by DnaC is blocked by interaction with YqaM.

The cI-like gene was found in the genomes of the prophage (-like) and prophage-related entities of *B. subtilis*, e.g., *c* ϕ 105 of prophage ϕ 105 (40), *yonR* of prophage SP β (16), *xre* of defective phage PBSX (19), and *immR* of mobile genetic element ICEBsI (1). Among these CI-like repressors, the binding regions of ϕ 105, Xre, and ImmR have been well studied. ϕ 105 and ImmR each bind to six operator (OR) sequences in their respective regulatory regions, and neither OR contains 2-fold symmetry (1, 40), whereas Xre binds to four ORs (O1 to O4) containing a 2-fold symmetric sequence, 5'-GATACAAA ATGTATC-3' (consensus of O1 to O4, underlined symmetric sequence), in the PBSX regulatory region. The sequences of the *skin* ORs also contain the 2-fold symmetric sequence 5'-G TAACGAATCGATAC-3' (in the case of OR2). We further found that the structures of the 2-fold symmetry of the ORs are important for recognition by SknR, which tightly binds and represses *yqaF-yqaN* expression through the three ORs (Fig. 4). The SknR protein may be able to cooperatively bind to these ORs by forming oligomers, but future studies will focus on defining these interactions.

The *yqaF* promoter is a strong promoter, as observed for

promoters of other genes involved in phage induction, leading to significant production of phage protein. The level of expression was examined for an ImmR-regulated gene, *xis*, which encodes an accessory protein of the site-specific recombinase in ICEBsI among the above-mentioned *B. subtilis* phage entities (1). The β -galactosidase activity for an ImmR-depleted strain harboring *Pxis-lacZ* showed almost the same level as that for an SknR-depleted strain harboring *PyqaF-lacZ* (Fig. 2B). The *yqaF* promoter sequence (5'-TTGACA-17 bp-TATTA-3') (Fig. 3A) is highly similar to that of *xis* (5'-TTGACT-17 bp-TATTA-3') (1), suggesting that both promoter sequences enable RNA polymerase to easily recognize them if the repressor is dissociated from the regulatory region. Significant expression of phage-related proteins that interact with host replication proteins can be reasonably expected to inhibit host cell growth.

Generally, prophage(-like) or prophage-related entities are induced by SOS-inducing agents such as UV irradiation and MMC treatment that could lead to DNA damage, which activates RecA-mediated proteolysis of the respective repressor protein. Such agents have been shown experimentally to induce the genomes of ϕ 105, SP β , PBSX, and ICEBsI (2, 20, 28, 29). However, Krogh et al. (12) reported previously that none of the *lacZ* fusions inserted into the *skin* element was expressed after exposing cells to MMC. Meanwhile, we also tested if the *skin* genes are under the control of the SOS response, but, consistent with the results of Krogh et al. (12), genes in the *skin* element did not have any apparent increased expression after MMC treatment (data not shown). SknR might evolve to be resistant to RecA-mediated proteolysis after the phage lost its ability to propagate.

Part of the *B. subtilis* *skin* element is preserved in *Clostridium difficile* (*skin*^{Cd}), whose *skin* element is also excised during sporulation by a site-specific recombinase and creates a new composite gene, *sigK* (8). However, Haraldsen and Sonenshein (8) showed interesting findings, in that (i) a *skin*-less *C. difficile* strain fails to complete sporulation, (ii) *skin*^{Cd} (14 kb) is approximately one-third the length of the *B. subtilis* *skin* element, (iii) *skin*^{Cd} lies farther downstream in the *sigK* gene than does *B. subtilis* *skin*, and (iv) the product of the *sigK* gene in *skin*^{Cd} has no pro-sequence (Pro- σ^K). Thus, Haraldsen and Sonenshein (8) proposed that the interruptions of *sigK* by prophage-like elements in *B. subtilis* and *C. difficile* represent independent events during evolution. In contrast, recently the genome sequence of *C. difficile* revealed that *skin*^{Cd} comprises a few phage related-genes, including a gene (CD1239 [35]) encoding a SknR homolog. There is no evidence that *C. difficile* SkinR is also resistant to RecA-mediated proteolysis, but it seems that in these two kinds of *skin* elements, mutations have debilitated some genes that cause adverse effects for the host cell (e.g., the cI-like gene region related to the induction mechanism for the SOS response; the genes like *yqaH* and *yqaM*, which are present in *B. subtilis* *skin* but missing for *C. difficile* *skin*) in the evolution of bacterial genomes.

ACKNOWLEDGMENTS

We thank M. Ogura for providing the pIS284 plasmid. We thank Norio Imaizumi and Akira Yoshinari for their technical assistance.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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